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Relationship Between a Stress Membrane Protein of *Oenococcus oeni* and Glyceraldehyde-3-Phosphate Dehydrogenases

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Abstract

The goal of this study was to analyze how the profiles of membrane proteins of *Oenococcus oeni* change under particular stress conditions of wine. Sodium dodecyl sulfate polyacrylamide gel electrophoresis protein profiles of membrane fraction showed that a 40-kDa protein was overexpressed in the presence of SO_2. The sequence of its N-terminal fragment showed a significant identity with glyceraldehyde-3-phosphate dehydrogenases (GAPDHs), but the protein showed no GAPDH activity. This sequence was compared with those of other GAPDHs with ClustalW alignment, and it was found to be somewhat similar to that of the cell-wall and membrane proteins of other lactic acid bacteria.

Index Entries: Glyceraldehyde-3-phosphate dehydrogenase; malolactic fermentation; *Oenococcus oeni*; stress; SO_2; wine.

Introduction

During the malolactic fermentation of wine, L-malic acid is decarboxylated to L-lactic acid and CO_2 by lactic acid bacteria, mainly *Oenococcus oeni*. As a consequence of their activity, the wine's quality improves, because the acidity is reduced; the organoleptic characteristics of the wine are enhanced;

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and the microbiologic stability of the wine increases *(1,2)*. However, *O. oeni* has to degrade L-malic acid under very unfavorable conditions such as low pH, high ethanol concentration and the presence of other compounds from the yeast metabolism *(3)*, and technological treatments such as SO_2 *(4)* or pesticides *(5)*.

Previous studies have shown that these stress conditions decrease *O. oeni* viability and delay malolactic fermentation *(6)*. In particular, it has been shown that SO_2 reduces the specific H^+-ATPase activity of *O. oeni (7)*. Moreover, the survival of *O. oeni* under stress conditions is related to the alteration of the structure and function of the membrane, among other reasons *(8)*. In this respect, electrophoretic protein profiles can show changes in membrane protein expression under stress conditions, which make cells more resistant to harsh conditions, even preventing cell death. Some proteins are newly synthesized or their expression levels are modified, and others are not expressed as well *(9)*. Among the stress proteins that can be induced by hostile conditions, heat-shock proteins have been well studied in *O. oeni (10,11)*.

In the present study, we analyzed the changes in profiles of *O. oeni* membrane proteins under the presence of SO_2, which acts as a stress condition at the levels used in the vinification process. We report the induction of a 40-kDa membrane protein in the presence of SO_2, a protein that has a significant similarity to proteins of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) family.

Materials and Methods

Chemicals

Pepstatin A, D,L-GAP, and monoclonal sheep antibody IgG-peroxidase conjugate were from Sigma-Aldrich (Steinheim, Germany). DNase I, lysozyme, NAD^+, and phenylmethylsulfonyl fluoride (PMSF) were from Roche (Basel, Switzerland).

Bacterial Strains and Growth Conditions

Two different strains of *O. oeni* were used: type culture CECT4100^T, obtained from the Colección Española de Cultivos Tipo (CECT) (Valencia, Spain); and CR1, isolated by our group from the Mas dels Frares winery (Rovira i Virgili University, Tarragona, Spain). These strains were cultivated at 27°C under anaerobic conditions in MRSMF medium, which consists of MRS *(12)* supplemented with D,L-malic acid (4 g/L) and fructose (5 g/L) at pH 5.0.

For assays of stress conditions, cells were grown in MRSMF up to the late log phase and incubated for 2 h in the presence of sulfite. Considering that levels of free molecular SO_2 ($SO_2 \cdot H_2O$)—the more active form of sulfite *(4,13)*—depend on the pH of the growth medium, SO_2 was added as $K_2S_2O_5$. The difference between the pH of wine in real conditions (3.2–3.8) and the

pH of the growth medium that we used here (5.0) was taken into consideration in order to achieve the same levels of $SO_2 \cdot H_2O$ (near 2.5%) present in wine-making conditions when 20 mg/L of SO_2 is added. This was determined by the constant of equilibrium corresponding to the reaction $SO_2 \cdot H_2O = H^+ + HSO_3^-$ (pK = 1.81). A concentration of 2.5% of free molecular SO_2 was confirmed in a parallel assay with enough volume to determine by volumetry with KI following García-Barceló *(14)*.

Isolation of Membrane and Cytoplasmic Fractions

Cells were suspended in 20 mL of 50 mmol/L Tris-HCl, 50 mmol/L sucrose buffer at pH 7.0 containing the following protease inhibitors: 1 mmol/L of PMSF and 3 µmol/L of pepstatin. Protoplasts were harvested, and membrane and cytoplasmic fractions were isolated following Rimpiläinen et al. *(15)* and Garbay and Lonvaud-Funel *(16)*. Samples were conserved at 4°C for the GAPDH assay and stored at –20°C for electrophoretic analysis.

GAPDH Activity Assay

GAPDH activity was measured following Ferdinand *(17)* and Pancholi and Fischetti *(18)* and was expressed as NADH produced (micromoles per minute per milligram of protein). Assays were done in triplicate.

Protein Electrophoresis and Western Blot Immunoassay

Proteins (100–500 µg) were extracted by precipitation with acetone from membrane and cytoplasmic fractions. Then they were solubilized in 10 mmol/L of Tris-HCl, 1 mmol/L of EDTA, 2.5% sodium dodecyl sulfate (SDS), 5.0% β-mercaptoethanol, and 0.01% bromophenol blue. Proteins were then denatured at 100°C for 5 min and separated by SDS-polyacrylamide gel electrophoresis (PAGE) using a Mini-Protean® II Cell System from Bio-Rad (Hercules, CA). They were detected by silver staining *(19)* and a low molecular weight marker from Amersham Biosciences was used for determination of molecular mass.

To perform a Western blot immunoassay *(20)*, the electrophoresed proteins were transferred to a polyvinyl difluoride (PVDF) membrane *(21)*. The location of the 40-kDa protein was determined with the polyclonal antibody against GAPDH derived from sheep, from Biogenesis (Poole, England). The protein-antibody interaction was detected by the peroxidase method, with a Sigma FAST DAB System (Sigma-Aldrich).

Analysis of Amino Acid Residue Sequence of Protein

The electrophoresed 40-kDa protein from the strain CR1 was transferred to a PVDF membrane *(21)*, and following Matsudaira *(22)*, its amino acid sequence in N-terminal residues was analyzed with the Edman degradation method in a Beckman LF3000 sequencer equipped with a phenylthiohydantoin (PTH)–amino acid analyzer (Protein Sequencing Ser-

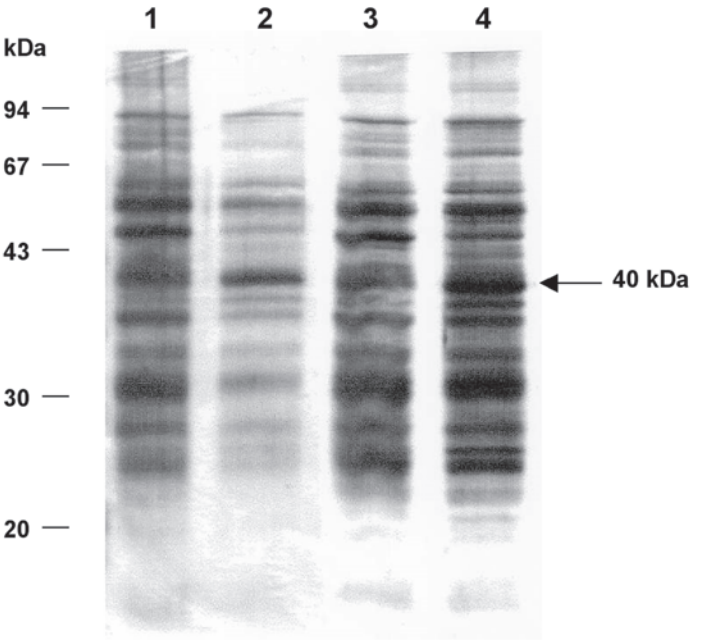

Fig. 1. SDS-PAGE of membrane fractions from strains of *O. oeni* CECT4100^T (lanes 1 and 2) and CR1 (lanes 3 and 4) incubated in presence of 0% (lanes 1 and 3) and 2.5% (lanes 2 and 4) free molecular SO_2. The molecular sizes corresponding to the low molecular weight marker are indicated on the left.

vice from the Institut de Biologia Fonamental, Universitat Autònoma de Barcelona).

Determination of Protein Concentration

Protein concentration was determined using a Bio-Rad protein assay kit *(23)*, with bovine serum albumin from Sigma as a standard.

Results

Protein profiles of *O. oeni* CECT4100^T and CR1 were analyzed in the presence of SO_2, considered an important stress condition. Membrane protein profiles experienced some changes. In particular, a protein close to 40 kDa was overexpressed under this condition in both strains (Fig. 1). By contrast, it can be seen that the synthesis of some other proteins was inhibited.

To obtain more information about the 40-kDa protein, its N-terminal fragment from the strain CR1 was sequenced (17 amino acid residues) (Table 1), and then it was aligned to the homologous sequences from the Swiss-Prot data bank with the WU-Blastp tool *(24)*. It showed a significant identity (70–82%) with the family of GAPDHs (EC 1.2.1.12), whose molecular weight is about 40 kDa.

Table 1
Comparison of N-terminal Amino Acid Sequence of 40-kDa Protein From *O. oeni* CR1 (This Work) With Sequences of GAPDH Obtained From Other Organisms With Which This Protein Shows Highest Identity, Indicated by Accession no. in Swiss-Prot Data Bank[a]

Protein	N-t sequence	Identity (%)[b]	Positives (%)[c]	Organism	Location[d]
	5 10 15 20 25				
40 kDa	-MVKIGINGFGXIXRLAF————————			*O. oeni* CR1	
Q9CDH4	MVVKVGINGFGRIGRLAFRRIQN———	76	88	*Lactococcus lactis* (subsp. *lactis*)	i
Q59906	-VVKVGINGFGRIGRLAFRRIQN———	76	88	*Streptococcus equisimilis*	ii
P50467	YVVKVGINGFGRIGRLAFRRIQN———	76	88	*Streptococcus pyogenes*	ii
Q9R5J2	-VVKVGINGFGRIGRLAFRRIQN———	76	88	*Streptococci* group A	iii
Q97NL1	MVVKVGINGFGRIGRLAFRRIQN———	76	88	*Streptococcus pneumoniae*	i
Q8VVB9	MVVKVGINGFGRIGRLAFRRIQN———	76	88	*Streptococcus thermophilus*	i
Q9ALW2	MVVKVGINGFGRIGRLAFRRIQN———	76	88	*Streptococcus agalactiae*	i
P52987	MVVKVGINGFGRIGRLALRRIQE———	75	87	*Lactococcus lactis* (subsp. *lactis*)	i
O32755	MTVKIGINGFGRIGRLAFRRIMDLGEE——	82	82	*Lactobacillus delbrueckii* (subsp. *bulgaricus*)	i
Q9R495	-VVKVGINGFGXIGXLAFDXIQN———	70	82	*Streptococcus pneumoniae*	iii
Q9L5X6	——KVGINGFGRIGRLAFRRIQN———	70	76	*Streptococcus gordonii*	iv
	*:****** * **:				

[a]The N-terminal sequences belong to the ClustalW alignment. The characters used by ClustalW format are defined as follows: asterisks indicate positions that have a single fully conserved residue; colons indicate fully conserved positions by "strong" related groups of amino acids.

[b]The percentage of conserved amino acids in identical positions.

[c]The percentage of amino acids with conserved physicochemical characteristics according to the WU-Blastp tool.

[d]The subcellular location where each protein was found: i, cytoplasmic; ii, membrane receptor; iii, surface; iv, extracellular location.

	total cell extract	sd1	cytoplasmic fraction	sd2	membrane fraction
T	8.272	0.29	8.384	0.3026	0.1
T SO_2	0.688	0.23	0.592	0.1597	0.1
CR1	9.509	0.07	11.809	1.374	0.1
CR1 SO_2	1.791	0.185	1.375	0.0875	0.1

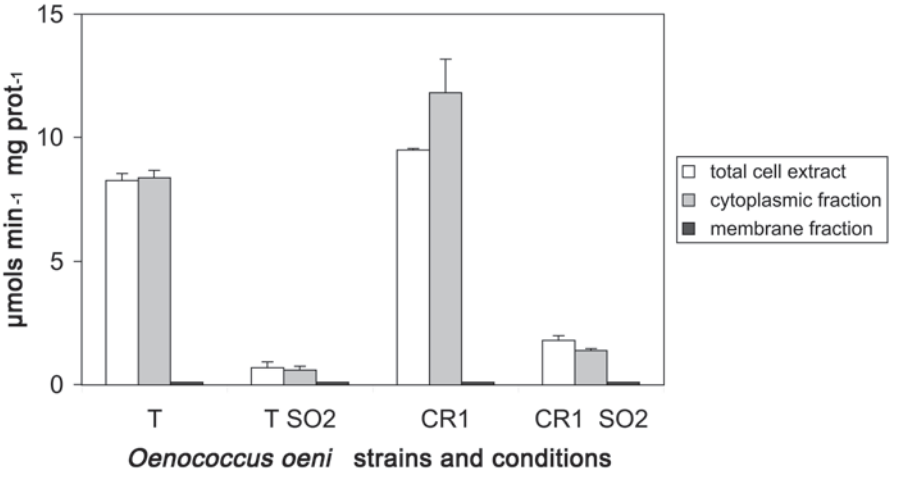

Fig. 2. Specific GAPDH activity in total cell extract and in cytoplasmic and membrane fractions from *O. oeni* strains CECT4100^T (T) and CR1. SO_2 here means 20 mg/L corresponding to 2.5% of free molecular SO_2. Bars represent the percentage of error (SD) for triplicate assays.

To determine whether the 40-kDa protein overproduced in *O. oeni* had a GAPDH activity, it was analyzed in the total cell extract and in cytoplasmic and membrane fractions in the presence of 2.5% free molecular SO_2 (Fig. 2). GAPDH activity was located only in the cytoplasmic fraction, and the values were very similar to the value for total cell extract. There was no activity in the membrane fraction. A Western blot assay of electrophoresed protein from the strain CR1 with the GAPDH antibody (tested with bacteria as *Bacillus stearothermophilus* and *Escherichia coli*) showed no significant binding with proteins from this fraction (data not shown). Moreover, cytoplasmic GAPDH specific activity was highly inhibited in the presence of SO_2 for the two strains studied, decreasing 16-fold in the presence of SO_2 in relation to the control for strain CECT4100^T, and 10-fold for strain CR1.

Thus, this 40-kDa membrane protein is homologous to GAPDH but apparently it does not show GAPDH activity. Analysis of WU-Blastp results showed that some membrane proteins that are homologous to GAPDH have additional functions. Table 1 shows a ClustalW alignment *(25)* of the 40-kDa N-terminal fragment of this protein with other GAPDH proteins that possess the highest levels of identity with the sequence found here. Some of those proteins are not located in the cytoplasm or have functions other than glycolytic activity.

Discussion

Enhanced expression of proteins as a response to stress environmental conditions has been described in *Lactococcus (26)*, *Lactobacillus (27)*, and also in *O. oeni (11,28)*. In the present study, we observed the overexpression of a 40-kDa protein in two strains of *O. oeni* in the presence of SO_2 (Fig. 1). At the same time, some of the other proteins were inhibited. In some cases, a decrease in protein synthesis in this species has also been described, particularly for acid shock *(10)*.

The N-terminal amino acid sequence of the 40-kDa protein from the strain CR1 was found to be homologous to that of the GAPDH family. GAPDH is an ubiquitous protein described in both prokaryotic and eukaryotic organisms, mainly as a glycolytic enzyme *(17)*, although certain species of GAPDHs have been found to be membrane bound *(29)*. GAPDHs showing major identity to this 40-kDa protein belong to other lactic acid bacteria (Table 1): *Streptococcus* (proteins P50467, Q9R5J2, and Q59906), *Lactobacillus* (protein O32755), and *Lactococcus* (proteins Q9CDH4 and P52987). The assays done to determine whether the 40-kDa protein had any GAPDH activity suggest that the lack of activity was not owing to a hypothetical SO_2 inhibition because no GAPDH activity was detected in control conditions (Fig. 2) and this evidence could not be explained only by normal expression of 40-kDa protein under no stress conditions. Moreover, this protein could also be considered totally unrelated to GAPDH, which would explain the activity results. The existence of GAPDH species that exhibit nonglycolytic activity or are placed in a noncytoplasmic location and do not display dehydrogenase activity has been described in some studies *(30)*. It could also be taken into account that GAPDH embedded in plasma membrane, as a tetramer composed of identical 40-kDa subunits, could suffer structural modifications leading to key alterations in catalytic domains involved in glycolytic activity.

Additionally, it was assumed that the cytoplasmic activity corresponded to the glycolytic enzyme. The glycolytic activity of GAPDH has been characterized in lactic acid bacteria, such as *L. lactis* spp. *lactis* IL1403 *(31)*. We have found a clear inhibition of cytoplasmic GAPDH activity by SO_2 in *O. oeni*. Although several investigators have studied the effect of SO_2 on the growth of lactic acid bacteria and malolactic fermentation *(7,32)*, no work has been done on how it affects a glycolytic enzyme such as GAPDH and, therefore, sugar catabolism.

Because this 40-kDa membrane protein did not show glycolytic activity, its sequence was compared with the sequences of those bacterial GAPDHs that had other additional functions and presented high levels of identity with the N-terminal sequence of the 40-kDa protein (Table 1). For instance, Pancholi and Fischetti *(18)* identified and purified a surface protein in *Streptococci* (Q9R5J2) that had dehydrogenase activity and a high capacity to bind eukaryotic proteins such as lysozyme, plasmin, fibronectin, and cytoskeleton proteins. Gase et al. *(33)* found a GAPDH protein in the

cell wall of *Streptococcus equisimilis* that bound plasmin and plasminogen (Q59906). Moreover, Kolberg and Sletten *(34)* described a 40-kDa protein (Q9R495) in the cell wall of *Streptococcus pneumoniae* that did not show GAPDH activity.

Further, Arnold and Pette *(35)* have already stated that glycolytic enzymes in eukaryotic cells may be associated with membranes or cytoskeleton structures. Glaser and Gross *(36)* purified in rat brain cytosol a fusogenic protein (i.e., a protein able to merge phospholipid bilayers) that was identified as a GAPDH (concretely two GAPDH isoenzymes were observed, one of them with nonglycolytic activity), and Sirover *(37)* described the involvement of human GAPDH in endocytosis and the export of nuclear tRNA.

Our study shows that changes took place in membrane proteins in two strains of *O. oeni* when they were grown under SO_2 stress conditions. The overproduction of a 40-kDa protein in the presence of SO_2 was shown, for the first time, in *O. oeni*. This membrane protein is homologous to GAPDH but it has no glycolytic activity. Its similarity to GAPDH proteins of other lactic acid bacteria with no glycolytic localization suggests that it may be involved in a diversity of functions related to environmental changes and cell membrane structure, helping the cell to survive in stress conditions. However, further advancements in the characterization of this protein require additional experiments.

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